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Preferential Selection of Human T-Cell Leukemia Virus Type 1 Provirus Lacking the 5′ Long Terminal Repeat during Oncogenesis▽

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In adult T-cell leukemia (ATL) cells, a defective human T-cell leukemia virus type 1 (HTLV-1) provirus lacking the 5′ long terminal repeat (LTR), designated type 2 defective provirus, is frequently observed. To investigate the mechanism underlying the generation of the defective provirus, we sequenced HTLV-1 provirus integration sites from cases of ATL. In HTLV-1 proviruses retaining both LTRs, 6-bp repeat sequences were adjacent to the 5′ and 3′ LTRs. In 8 of 12 cases with type 2 defective provirus, 6-bp repeats were identified at both ends. In five of these cases, a short repeat was bound to CA dinucleotides of the *pol* and *env* genes at the 5′ end, suggesting that these type 2 defective proviruses were formed before integration. In four cases lacking the 6-bp repeat, short (6- to 26-bp) deletions in the host genome were identified, indicating that these defective proviruses were generated after integration. Quantification indicated frequencies of type 2 defective provirus of less than 3.9% for two carriers, which are much lower than those seen for ATL cases (27.8%). In type 2 defective proviruses, the second exons of the *tax*, *rex*, and *p30* genes were frequently deleted, leaving Tax unable to activate NF-κB and CREB pathways. The *HTLV-1 bZIP factor* gene, located on the minus strand, is expressed in ATL cells with this defective provirus, and its coding sequences are intact, suggesting its significance in oncogenesis.

Human T-cell leukemia virus type 1 (HTLV-1) is the causative virus of a neoplastic disease, adult T-cell leukemia (ATL), and inflammatory diseases, including HTLV-1-associated myelopathy/tropical spastic paraparesis and HTLV-1-associated uveitis (18, 32). After a long latent period, about 5% of carriers develop ATL (3). These observations suggest that several factors, including the host immune system, genetic background, and viral genes, influence ATL onset.

Retroviruses induce cancers by several different mechanisms (37), including viral oncogenes (51), aberrant transcription of oncogenes by insertion of provirus (24), and actions of virus-encoded genes (14, 55). HTLV-1 is a complex retrovirus, which encodes regulatory and accessory genes in the pX region by four open reading frames. Among those, *tax* is thought to play a central role in both immortalization and oncogenesis by pleiotropic activity (15, 21, 55). Tax activates the transcription of cellular genes, via nuclear factor κB (NF-κB), cyclic AMP response element-binding protein (CREB), and activator protein-1 pathways, and can inhibit functions of cellular proteins, including p53 and MAD1. In addition, the plus strand of the pX region encodes accessory genes *p12*, *p13*, and *p30* in the different open reading frames (39). The p12 protein interacts with calreticulin and calnexin (11) and enhances nuclear factor of activated T cells (NFAT)-dependent gene expression (2, 10, 28). The p30 protein binds to CREB binding protein/p300 and modulates the transcription of viral and cellular genes (33, 56). These accessory genes are required for infectivity and for the persistence of HTLV-1 infection (1, 49). Tax and accessory gene products therefore increase the number of HTLV-1-infected cells by promoting proliferation and inhibiting apoptosis. On the other hand, Tax expression makes infected cells a major target of cytotoxic T lymphocytes (CTLs) in vivo, thereby decreasing their number (6). CTLs against p12 and p13 have been also reported in HTLV-1 carriers (43). Thus, proliferation of HTLV-1-infected cells is determined by a balance in activities between viral genes and the host immune system. On the other hand, HTLV-1 has redundant mechanisms to suppress Tax expression. Rex suppresses the transport of doubly spliced viral RNA (25) encoding Tax and Rex. p30 binds to *tax* gene transcripts and inhibits their transport into the cytoplasm (38). In addition, HTLV-1 bZIP factor (HBZ) suppresses viral gene transcription through the 5′ long terminal repeat (LTR) by binding to c-Jun, a transcription factor critical for *tax* expression (7).

Tax expression in primary leukemic cells is absent in about 60% of ATL cases. Three mechanisms to suppress or abolish Tax expression have been identified (31). Genetic changes in *tax* are seen in about 10% of ATL cases (17, 52). DNA methylation of the 5′ LTR also silences *tax* transcription (29, 52, 54). In addition, the 5′ LTR of HTLV-1, which functions as a promoter/enhancer, is frequently deleted in ATL cases, and such deletion results in loss of viral gene transcription unless the defective provirus traps a cellular promoter. This type of provirus is designated the type 2 defective provirus, and its frequency is higher in aggressive forms of ATL than in chronic or smoldering ATL (53). However, it remains unclear when deletion occurs in the course of disease progression. Although the 5′ LTR is frequently methylated or deleted, the 3′ LTR remains intact in ATL cells. *HBZ* is transcribed from the minus

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strand of the provirus using the 3′ LTR as a promoter. We have reported that *HBZ* is expressed in ATL cells and promotes ATL cell proliferation (44). *HBZ* suppresses *tax* gene transcription through the 5′ LTR, simultaneously supporting the growth of ATL cells.

After infection, retroviruses synthesize a double-stranded proviral DNA by using reverse transcriptase (RT) with tRNA as a primer. Double-stranded viral DNA is then integrated by viral integrase and cellular factors (5, 20). During this process, integrase generates short repetitive sequences of 4 to 6 bp adjacent to both LTRs. The length of these repeats is specific to each retrovirus. For example, human immunodeficiency virus type 1 forms a 5-bp short repeat at the ends of both LTRs, and murine leukemia virus generates 4-bp repeats. Identification of these short repeats indicates that integration is mediated by viral integrase.

In this study, we determined the integration sites and neighboring genomic sequences of the HTLV-1 provirus and found that 6-bp repeat sequences adjacent to both ends of the type 2 defective provirus were retained in 8 of 12 cases. This finding indicates that provirus lacking a 5′ LTR can be integrated by the viral integrase and that deletion of the 5′ LTR occurs before integration. In the remaining four cases, the short repeat was absent and host genomic sequences were deleted, indicating that these defective proviruses formed after integration. In type 2 defective proviruses, *HBZ* sequences were conserved, and *HBZ* transcripts were detected in ATL cells with type 2 defective provirus, suggesting that they are functional in such ATL cells.

MATERIALS AND METHODS

Samples and cell lines. Clinical samples were collected from 79 ATL patients after informed consent was obtained. Clinical diagnosis of ATL subtypes was performed according to criteria reported previously (47). Approval for this study was obtained from the institutional review board of Kyoto University. Genomic DNA was extracted from peripheral blood mononuclear cells or lymph node cells using standard phenol-chloroform methods. The ATL cell lines TL-Om1 and ATL-55T were used in this study.

Determination of proviral subtypes by PCR and Southern blotting. Subtypes of HTLV-1 provirus were determined as reported previously (53). In brief, the whole HTLV-1 provirus was amplified by long PCR using primer 1 (5′-GTTCC ACCCCTTTCCCTTTCATTCACGACTGACTGC-3′) and primer 2 (5′-GGCT CTAAGCCCCCGGGGGATATTTGGGGCTCATGG-3′). PCR conditions were as follows: denaturation at 94°C for 30 s and annealing and extension at 64°C for 10 min; 30 cycles were run. The pX region of the provirus was amplified by primer 2 and primer 3 (5′-GGCGACTGGTGCCCCATCTCTGGGGGACT ATGTTCG-3′). As a control, we used the genomic DNAs from HTLV-1 carriers and confirmed that there was no band with this condition. When the whole provirus was amplified, the complete provirus (7.7 kb) could be detected using primers 1 and 2, while type 1 defective provirus generated a smaller band. In type 2 defective provirus, no band was amplified. PCR products (1.0 kb) derived from the pX region were detected in all cases by primers 2 and 3. Southern blotting was performed as described previously (53).

Inverse PCR. Genomic regions flanking the 3′ LTR of the HTLV-1 provirus were amplified by inverse PCR as described previously (13). Conditions for long PCR were as follows: 1 cycle of 98°C for 2 min, 5 cycles of 98°C for 30 s and 64°C for 10 min, and 35 cycles of 94°C for 30 s, 64°C for 10 min, and 72°C for 15 min. Primers were as follows: long-IPCR-F (5′-TGCCTGACCCTGCTTGCTCAAC TCTACGTCTTTG-3′) and long-IPCR-R (5′-AGTCTGGGCCCTGACCTTTT CAGACTTCTGTTTC-3′), and pX-3′ F1 (5′-TGGCACGCCTATGATTTCCG-3′) and pX-R1-Ban III (5′-GGGGGTTGTATGAGTGATTGG-3′).

Sequencing of genomic regions adjacent to integration sites and proviruses. Inverse-PCR products from ATL samples were used as templates for direct sequencing with a primer located at the end of the 3′ LTR (5′-GTTCCACCC CTTTCCCTTTCATTCACGACTGACTGC-3′). After integration sites were determined, the 5′ ends of the integration sites were amplified by PCR using primers based on 5′ human genomic regions at integration sites and based on internal provirus regions. Internal regions of type 2 defective provirus were also amplified by PCR using primers based on *gag*, *pol*, *env*, and the 3′ LTR, and their sequences were determined by direct sequencing. Sequencing was performed using the Big Dye Terminator (version 3.1) cycle sequencing kit and an ABI310 autosequencer (both from Applied Biosystems, Foster City, CA).

RT-PCR. Spliced *HBZ* and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) transcripts were detected by RT-PCR using a PC-808 thermal cycler (Astec, Fukuoka, Japan) under the following conditions: 1 cycle of 95°C for 2 min, followed by 35 cycles of 95°C for 30 s, 57.5°C for 30 s, and 72°C for 30 s. The primers used were as follows: 5′-TAAACTTACCTAGACGGCGG-3′ and 5′-C TGCCGATCACGATGCGTTT-3′ for the *HBZ* gene and 5′-GCAGGGGGGA GCCAAAAGGG-3′ and 5′-TGCCAGCCCCAGCGTCAAAG-3′ for *GAPDH*. As a positive control for *HBZ* gene transcription, we used mRNA derived from an ATL cell line, ATL-55T.

Quantification of type 2 defective provirus in carriers. First, genomic DNA from a single ATL sample with one type 2 defective provirus was mixed in various proportions (5, 10, 20, 40, and 100%) with DNA from an ATL cell line harboring a complete provirus (TL-Om1). Then we used real-time PCR to quantify provirus loads at three different regions of the provirus: (i) 5′ LTR-*gag* (deleted only in type 2 defective provirus), (ii) *gag* (deleted in both type 1 and type 2 defective proviruses), and (iii) pX (conserved in all proviruses). Primer pairs were 5′LTR-SDS-F (5′-AA GTACCGGCGACTCCGTTG-3′) and *gag*-SDS-R (5′-AGCGCTACGGGAAAA GATTTG); *gag*-F (5′-GAGTGCCAAAGACCCTTCCT-3′) and *gag*-R (5′-GTCA AGAGCTATGTTGAGGCG-3′); and *tax*-exon3-F (5′-GAAGACTGTTTGCCCA CCACC) and *tax*-exon3-R (5′-TGAGGGTTGAGTGGAACGGA). Probes were the 5′ LTR probe (5′-CGTCCGGGATACGAGCGCCCCTT-3′), the *gag* probe (5′-CAAGGCCTGGAGGAGCCTTACCACG-3′), and the pX probe (5′-CACCC GTCACGCTAACAGCCTGGCAA-3′). Real-time PCR was performed using an ABI Prism 7700 sequence detection system, Taqman Universal PCR master mix, and genomic DNAs (200 ng). PCR amplification consisted of 1 cycle of 50°C for 2 min, 1 cycle of 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min. All samples were analyzed in triplicate. Provirus load is shown as the percentage of mononuclear cells that are infected, assuming that one infected cell contains one copy of HTLV-1 provirus.

Following quantification of provirus loads at 5′ LTR-*gag* and pX, regression lines were drawn using the least-squares method to determine the frequency of type 2 defective provirus. The frequency of defective provirus (type 1 plus type 2) was calculated from provirus loads at the *gag* and pX regions. We examined the genomic DNAs of two carriers with high proviral loads (64.2% for carrier 1 and 47.2% for carrier 2).

3′-RACE. To determine the 3′ ends of the *HBZ* transcripts, rapid amplification of cDNA ends (RACE) was performed using the SMART RACE cDNA amplification kit (BD Biosciences Clontech), according to the manufacturer's protocol. First-strand cDNAs were synthesized from 1 μg of total RNA with RT and were used for 3′-RACE PCR. For nested 3′-RACE amplifications, primers specific for *HBZ* (5′-CTAGGTTAGGGCAGGGGGGCTGTAGGGC-3′ and 5′-GGGTCCACGAACAAACTGGCTGGGCAGG-3′) were used. The sequence of the PCR product was determined by direct sequencing.

Mapping of HTLV-1 integration sites and expression of trapped genes. The BLAT program was used to map identified integration sites in the human genome (UCSC Human Genome Project Working Draft, May 2006 freeze). Sequences were judged authentic only if they showed 95% or higher identity to genomic sequence over the high-quality sequence region and matched only one genomic locus with 95% or greater identity. Gene expression was evaluated by the GeneCards database of the Crown Human Genome Center at the Weizmann Institute of Science (http://www.genecards.org/background.shtml).

RESULTS

Genomic sequences of HTLV-1 integration sites. Three subtypes of HTLV-1 provirus are seen in ATL cells: the complete type, the type 1 defective type, and the type 2 defective type, as shown in Fig. 1A. Type 1 defective provirus retains the 5′ and 3′ LTRs but lacks internal sequences such as the *gag*, *pol*, or *env* region, whereas type 2 defective provirus lacks the 5′ LTR in addition to internal sequences. We determined the subtypes of HTLV-1 provirus in 79 ATL cases by long PCR (Fig. 1B) and by Southern blotting as reported previously (53). Among samples evaluated, type 2 defective proviruses were found in

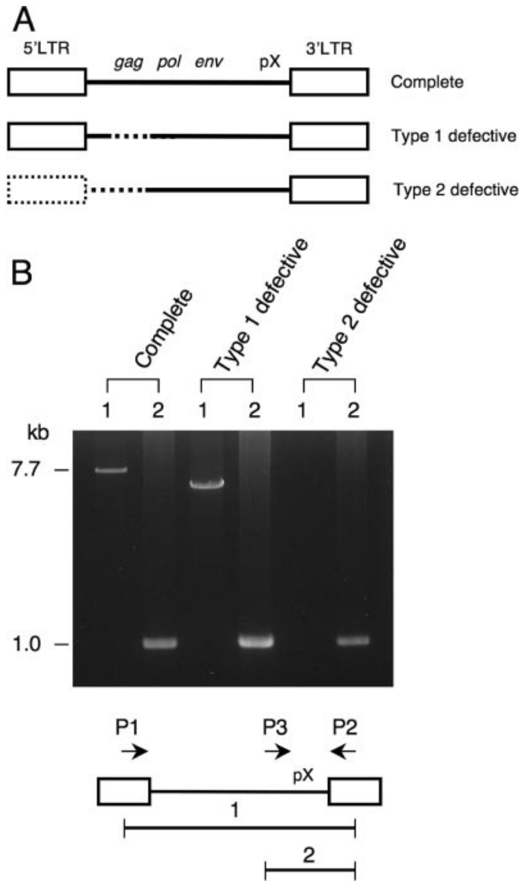

FIG. 1. Three subtypes of HTLV-1 provirus in ATL cells. (A) Schemas of HTLV-1 proviruses in ATL. Dotted lines represent deleted regions of HTLV-1 provirus. (B) Representative results of PCR in three subtypes of HTLV-1 proviruses. Whole HTLV-1 provirus was amplified using LTR primers (primers 1 and 2 [P1 and P2] as described in Materials and Methods). The 7.7-kb band (band 1) was detected in the case with complete provirus. In type 1 defective provirus, the smaller band was amplified by primers 1 and 2. In type 2 defective provirus, no band was detected. On the other hand, the 1.0-kb band (band 2) derived from pX-3′ LTR was amplified in all cases.

27.8% of the cases, while the frequency of type 1 defective proviruses was 11.4% (Table 1).

Genomic regions adjacent to HTLV-1 integration sites were amplified by inverse PCR as described in Materials and Methods, and their sequences were determined. Six-base-pair repeats and CA dinucleotides at the ends of both LTRs were conserved in all cases with complete or type 1 defective provirus (cases 1 to 15) (Fig. 2A). These results indicate that these proviruses were likely integrated by the retrovirus integrase.

Among 22 cases with type 2 defective provirus, we identified genomic sequences at integration sites in 12 cases, due to limited availability of DNA samples. Among those 12 cases, 6-bp repeats adjacent to the provirus sequence were observed in 8 cases (cases 16 to 23) (Fig. 2A). These cases could be divided into two groups. In the first, the 5′ LTR was completely deleted and TG dinucleotides in the *pol* and *env* regions were directly ligated to a 6-bp repeat of the host genome (cases 16 to 20), indicating that these proviruses were integrated by viral integrase. In these cases, the viral integrase likely recognized a TG sequence in the *pol* or *env* region, indicating that the integrase can recognize only a TG dinucleotide for activity. In the second group, a short fragment of the 5′ LTR (18 to 43 bp) remained, but most of the LTR was deleted, in addition to the *gag* and *pol* regions (cases 21 to 23). It is not clear whether these regions were deleted before or after integration. In two cases, inverted provirus sequences were observed. In case 20, an inverted *gag* and *pol* sequence was ligated to the 6-bp repeat at the 5′ end, and a TG dinucleotide was retained. In case 23, *pol* sequences were inverted and adjacent to the short U3 sequence.

In four cases of type 2 defective provirus (cases 24 to 27 in Fig. 2A), there was no short repeat sequence adjacent to the provirus, and loss of host genomic sequence (6 to 26 bp) was also seen at the integration site (Fig. 2B). In addition, 2- to 4-bp overlaps were identified at breakpoints, features that are characteristic of illegitimate recombination (50). In addition, the 3′ end of the 3′ LTR retained CA sequences in all cases. These observations suggest that this provirus is first integrated by the viral integrase, and then proviral sequences, including the 5′ LTR and host genomic sequences, are deleted.

Structure of regulatory and accessory genes in type 2 defective provirus. We determined the entire sequence of type 2 defective proviruses in 12 ATL cases by PCR and sequencing (Fig. 3). Although type 2 defective provirus lacks a viral promoter/enhancer (5′ LTR), such a provirus might trap a cellular promoter. The integration sites, trapped cellular genes, and prospective transcription of viral genes are summarized in Table 2. Among five proviruses integrated in a transcriptional unit, the direction of viral transcription matched that of cellular genes in all cases. By use of the GeneCards database (http://www.genecards.org/background.shtml), four of these genes (DONSON, PCDH9, ALPK1, and CLEC7A) were found to be ubiquitously expressed, and the fifth gene, XYLT1, was transcribed in the heart, spleen, and brain. In three of these cases (cases 16, 18, and 21), the second exon of the *tax*, *p30*, and *rex* genes was retained, indicating that these genes could be transcribed in these ATL cells. Among type 2 defective proviruses, the second exon of the *tax*, *rex*, and *p30* genes was deleted in seven cases (cases 17, 20, 22, 23, 24, 25, and 27 [Fig. 3]). Without this exon, Tax protein lacks NF-κB- and CREB-activating activities (48). Taken together, these data indicate that wild-type Tax proteins cannot be produced in nine cases due to

TABLE 1. Subtypes of HTLV-I provirus in ATL cases

Type of provirus[a]	No. (%) of cases	No. of cases with the following clinical type of ATL:		
		Acute	Chronic	Lymphoma
Complete	43 (54.4)	31	8	4
Defective				
Type 1	9 (11.4)	4	4	1
Type 2	22 (27.8)	18	2	2
Multiple types	5 (6.3)	4	1	0
Total	79 (100)	57	15	7

[a] Subtypes of HTLV-I provirus in ATL have been determined by PCR and Southern blotting as reported previously (54). Multiple types have more than 2 copies of provirus per cell.

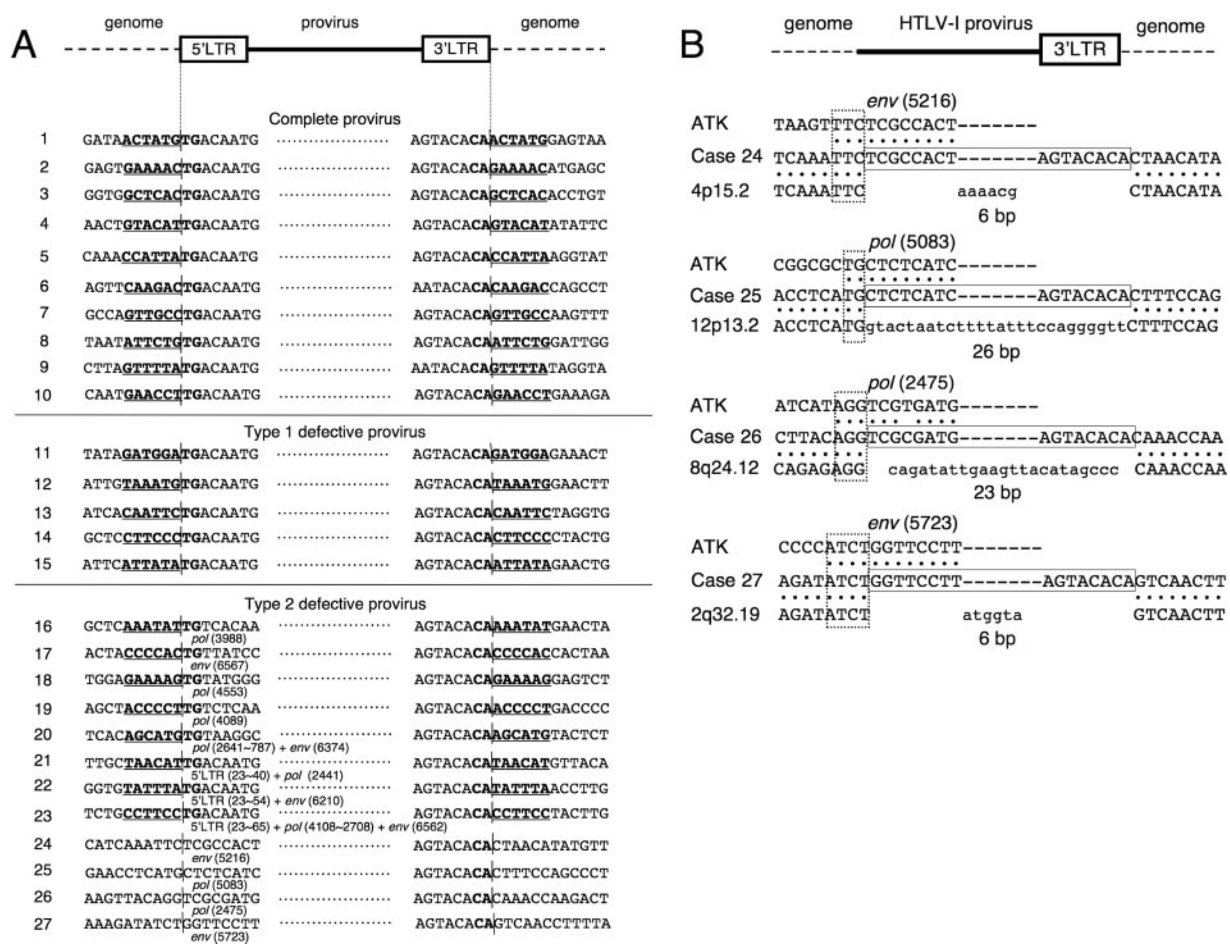

FIG. 2. Genomic sequences adjacent to HTLV-1 integration sites in ATL cells. (A) Neighboring genomic regions were amplified by inverse PCR and sequenced. The 6-bp short repeats at the 5′ and 3′ ends of proviruses are underlined. The 6-bp repeats and 2 bp of adjacent LTRs are boldfaced. In type 2 defective proviruses, the position of the deletion in the provirus, which is adjacent to the human genomic sequence, is shown using the numbering system of Seiki et al. (46) (GenBank accession no. J02029). Vertical bars show boundaries between genomic sequences and the provirus. (B) Type 2 defective proviruses lacking the 6-bp repeat. In each case, the sequences from the HTLV-1 provirus (top) (ATK-1 [46]), the integrated provirus (center), and the host genome (bottom) are compared. Proviral sequences are boxed by solid lines. Deleted genomic sequences are lowercased. The chromosomal locations of integration sites are shown. At the 5′ breakpoints, overlapped regions (2 to 4 bp) between the provirus and the genome are boxed by dotted lines.

either deletion of an exon or lack of a promoter, while in three cases type 2 defective provirus can generate Tax, Rex, p12, p13, and p30 proteins by using cellular promoters.

***HBZ* coding sequences in type 2 defective proviruses.** Recently, we reported that *HBZ* was transcribed in all ATL cases examined and that it supported the growth of ATL cells, suggesting that it is critical for leukemogenesis (44). Examination of sequences of *HBZ* in type 2 defective proviruses revealed no nonsense mutations, insertions, or deletions, although several missense mutations were detected (Fig. 4 and 5). Two *HBZ* polyadenylation signal sequences have been identified (8, 35, 44), both deleted in case 17, as shown in Fig. 6B. Next, we examined *HBZ* transcription in ATL cells with type 2 defective proviruses. In four cases in which RNAs were available for analysis, *HBZ* was transcribed in all cases, including case 17 (Fig. 6A). We determined the 3′end of the *HBZ* transcript in case 17 by 3′-RACE and found that the *HBZ* gene trapped a cellular polyadenylation signal (Fig. 6B). In case 17, the proviral sequence recombined with a human genomic sequence (7q21) in the *env* region (nucleotide 6567 according to the numbering of Seiki et al. [46]). The polyadenylation signal (attaaa [underlined in Fig. 6B]) in the host genome sequences was detected upstream of the poly(A) tail.

Frequencies of defective proviruses in HTLV-1 carriers. As shown in Table 1, the frequencies of type 1 and type 2 defective proviruses in ATL cases were 11.4% and 27.8%, respectively. However, their frequencies in the carrier state were unknown. To estimate those frequencies, we quantified provirus loads at three different regions of the provirus, including the region conserved in all types of provirus (pX), the region deleted in both types of defective provirus (*gag*), and the region deleted only in type 2 defective provirus (5′ LTR-*gag*). We determined that part of the *gag* region (nucleotides 1591 to 1681 according to the numbering of Seiki et al. [46]) was deleted in 95% of defective proviruses (data not shown). The 5′ LTR-*gag* region was absent only in type 2 defective provirus, whereas *gag* nucleotides 1591 to 1681 were deleted in both type 1 and type 2 proviruses. By contrast, the pX region was present in all pro-

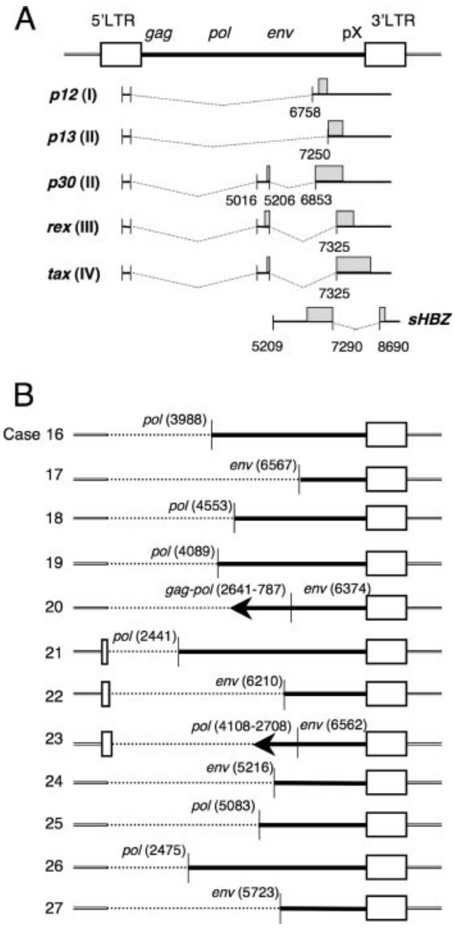

FIG. 3. Schematic diagram of type 2 defective proviruses. (A) The splicing patterns of the *p12*, *p13*, *p30*, *rex*, *tax*, and *HBZ* genes are indicated. Hatched boxes represent coding regions. The number of open reading frames (I, II, III, or IV) in the pX region is given in parentheses. Numbering is according to the work of Seiki et al. (46). (B) For cases with type 2 defective proviruses, dotted lines represent deleted portions, and arrows (cases 20 and 23) indicate inverted regions of proviruses. Positions of defective proviruses correspond to those of the wild-type virus diagramed in panel A. Type 2 defective provirus lacks the second exon of *tax* in 7 of 12 cases (cases 17, 20, 22, 23, 24, 25, and 27). In cases 21, 22, and 23, parts of the 5′ LTR remain.

viruses. As controls, we mixed various proportions of genomic DNA derived from ATL cases exhibiting type 2 defective provirus with DNA from an ATL cell line, TL-Om1, carrying the complete provirus, in various proportions, i.e., 0, 5, 10, 20, and 40%. Based on data for provirus loads from these samples, regression lines were drawn using the least-squares method (Fig. 7). Evaluation of provirus loads at the 5′ LTR-*gag* and pX regions enabled us to estimate the frequency of type 2 defective provirus (Fig. 7A). Quantification of the *gag* and pX regions indicated the frequencies of total defective (type 1 plus type 2) provirus (Fig. 7B). We measured provirus loads at three provirus regions using DNA samples from two carriers with high provirus loads (64.2% in carrier 1, and 47.2% in carrier 2) and then calculated the frequencies of type 2 and total defective proviruses (Fig. 7A and B). Due to the limitations of this assay, we could not determine the frequencies of defective provirus in carriers with moderate or low provirus loads. The results showed that the frequencies of type 2 defective provirus in the two carriers were 3.9 and −0.6%. Likewise, the frequency of total defective provirus was 11.8% in carrier 1 and 12.7% in carrier 2. These results suggest that the frequency of type 2 defective provirus in the carrier state is much lower than that in ATL cases, while the frequencies of type 1 defective provirus in ATL cases and the carrier state seem to be similar.

DISCUSSION

A defective provirus lacking the 5′ LTR has been reported in B-cell neoplasms caused by the avian leukosis virus (ALV). In these tumors, defective ALV proviruses were integrated into the 5′ region of the c-*myc* gene, and the 3′ LTR drove expression of c-*myc* (16, 36, 41). The 5′ LTR causes transcriptional interference (9) and is deleted in tumors induced by ALV. In contrast to ALV, HTLV-1 provirus integration sites are random in ATL cells (12, 40, 45), indicating that the 3′ LTR does not activate the transcription of specific cellular genes. By contrast, we have reported that the *HBZ* gene, which is transcribed from the 3′ LTR, is required for the proliferation of ATL cells (44). The observations that *HBZ* is transcribed in ATL cells with type 2 defective proviruses and that the *HBZ*

TABLE 2. Predicted transcription of regulatory and accessory genes in type 2 defective proviruses

Case no.	Coding gene	Transcriptional direction	Integration site	Predicted transcription[a] of the following gene:						Clinical subtype
				p12	*p13*	*p30*	*tax*	*rex*	*HBZ*	
16	XYLT1	Sense	16p12	+	+	+	+	+	+	Acute
17	None		7q21	−	−	−	−	−	+	Acute
18	DONSON	Sense	21q22	+	+	+	+	+	+	Acute
19	None		4p15	−	−	−	−	−	+	Acute
20	None		1q12	−	−	−	−	−	+	Lymphoma
21	PCDH9	Sense	13q21	+	+	+	+	+	+	Acute
22	None		5q23	−	−	−	−	−	+	Acute
23	ALPK1	Sense	4q25	+	+	−	−	−	+	Chronic
24	None		4p15	−	−	−	−	−	+	Acute
25	CLEC7A	Sense	12p13	+	+	−	−	−	+	Lymphoma
26	None		8q24	−	−	−	−	−	+	Acute
27	None		2q32	−	−	−	−	−	+	Acute

[a] The transcription of regulatory (*tax* and *rex*) and accessory genes (*p12*, *p13*, *p30*, and *HBZ*) is predicted from data on integration sites and sequences. +, possible transcription; −, transcription is not expected by loss of promoters or exons. In five cases, cellular gene promoters are trapped (cases 16, 18, 21, 23, and 25). Transcriptional units near integration sites were analyzed by the BLAT program (UCSC Human Genome Project Working Draft, May 2006 freeze).

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ATK  atggcggcctcagggctgtttcgatgcttgcctgtgtcatgcccggaggacctgctggtggaggaattggtggacgggctattatccttggaggaagagt  100
16   ----------------------------------------------------------------------------------------------------  100
17   ----------------------------------------------------------------------------------------------------  100
18   ----------------------------------------------------------------------------------------------------  100
19   --------------------------------g-------------------------------------------------------------------  100
20   ----------------------------------------------------------------------------------------------------  100
21   ------------------------------------c---------------------------------------------------------------  100
22   ----------------------------------------------------------------------------------------------------  100
23   ----------------------------------------------------------------------------------------------------  100
24   ----------------------------------------------------------------------------------------------------  100
25   ----------------------------------------------------------------------------------------------------  100
26   ---------------------------------a--c---------------------------------------------------------------  100
27   ----------------------------------------------------------------------------------------------------  100

ATK  taaaggacaaggaggaggaggaagctgtgcttgacggtttgctatccttagaagaggaaagccgcggccggctgcgacggggccctccaggggagaaagc  200
16   ----------------------------------------------------------------------------------------------------  200
17   ----------------------------------------------------------------------------------------------------  200
18   ----------------------------------------------------------------------------------------------------  200
19   ----------------------------------------------------------------------------------------------------  200
20   ---------------------------------------------tt-----------------------------------------------t-----  200
21   ----------------------------------------------------------------------------------------------------  200
22   ----------------------------------------------------------------------------------------------------  200
23   ----------------------------------------------------------------------------------------------------  200
24   ----------------------------------------------------------------------------------------------------  200
25   ----------------------------------------------------------------------------------------------------  200
26   ----------------------------------------------------------------------------------------------------  200
27   ----------------------------------------------------------------------------------------------------  200

ATK  gccacctcgcggggaaacgcatcgtgatcggcagcgacgggctgaggagaagaggaagcgaaaaaaagagcgggagaaagaggaggaaaagcagaccgct  300
16   -----------------------------------------------------a----------------------------------------------  300
17   ----------------------------------------------------------------------------------------------------  300
18   ----------------------------t------------------------------a----------------------------------------  300
19   -----------------------------------------------------------------------------------------------tt---  300
20   ----------------------------------------------------------------------------------------------------  300
21   ----------------------------------------------------------------------------------------------------  300
22   -------t--------------------------------------------------------------------------------------------  300
23   ----------------------------------------------------------------------------------------------------  300
24   ----------------------------------------------------------------------------------------------------  300
25   ----------------------------------------------------------------------------------------------------  300
26   --------------------------------------a-------------------------------------------------------------  300
27   ----------------------------------------------------------------------------------------------------  300

ATK  gagtatttgaaaaggaaggaagaggagaaggcacggcgcaggaggcgggcggagaagaaggccgctgacgtcgctaggaggaagcaggaagagcaggagc  400
16   --------------------------------------------------------------------------c-------------------------  400
17   --------------------------------------------------------------------------c-------------------------  400
18   --------------------------------------------------------------------------c-------------------------  400
19   --------------------------------------------------------------------------c-------------------------  400
20   --------------------------------------------------------------------------c-------------------------  400
21   --------------------------------------------------------------------------cc----a-------------------  400
22   --------------------------------------------------------------------------c-------------------------  400
23   --------------------------------------------------------------------------c-------------------------  400
24   ----------g---------------------------------------------------------------c-------------------------  400
25   --------------------------------------------------------------------------c-------------------------  400
26   --------------------------------------------------------------------------cc-a--a-------------------  400
27   --------------------------------------------------------------------------c-------------------------  400

ATK  gccgtgagcgcaagtggagacaaggggctgagaaggcgaaacagcatagtgctaggaaagaaaaaatgcaggagttggggattgatggctatactagaca  500
16   ----------------------------------------------------------------------------------------------------  500
17   ----------------------------c-----------------------------------------------------------------------  500
18   ----------------------------------------------------------------------------------------------------  500
19   ----------------------------------------------------------------------------------------------------  500
20   ----------------------------------------------------------------------------------------------------  500
21   ----------------------------------------------------------------------------------------------------  500
22   ----------------------------------------------------------------------------------------------------  500
23   ----------------------------------------------------------------------------------------------------  500
24   ----------------------------------------------------------------------------------------------------  500
25   ----------------------------------------------------------------------------------------------------  500
26   ----------------------------------------------------------------------------------------------------  500
27   ----------------------------------------------------------------------------------------------------  500

ATK  gttggaaggcgaggtggagtccttggaggctgaacggaggaagttgctgcaggagaaggaggatttgatgggagaggttaattattggcaggggaggctg  600
16   ----------------------------------------------------------------------------------------------------  600
17   ------------------------------------------------------t---------------------------------------------  600
18   ----------------------------------------------------------------------------------------------------  600
19   ----------------------------------------------------------------------------------------------------  600
20   ------------------------------------------------------t---------------------------------------------  600
21   ------------------------------------------------------------------a---------------------------------  600
22   ----------------------------------------------------------------------------------------------------  600
23   ------------------------------------------------------t---------------------------------------------  600
24   ----------------------------------------------------------------------------------------------------  600
25   ----------------------------------------------------------------------------------------------------  600
26   ------------------------------------------------------------------a---------------------------------  600
27   ----------------------------------------------------------------------------------------------------  600

ATK  gaggcgatgtggttgcaataa  621
16   ---------------------  621
17   c--------------------  621
18   ---------------------  621
19   ---------------------  621
20   c--------------------  621
21   ---------------------  621
22   ---------------------  621
23   c--------------------  621
24   ---------------------  621
25   ---------------------  621
26   ---------------------  621
27   ---------------------  621
```

FIG. 4. Nucleotide sequences of the *HBZ* gene in ATL cells with type 2 defective provirus. The coding sequences of the *HBZ* gene in type 2 defective proviruses were amplified, and their sequences were determined. Case numbers are given on the left. The nucleotide sequence of the *HBZ* gene in ATK-1 is shown as a control.

```
ATK  MAASGLFRCLPVSCPEDLLVEELVDGLLSLEEELKDKEEEEAVLDGLLSLEEESRGRLRR  60
16   ------------------------------------------------------------  60
17   ------------------------------------------------------------  60
18   ------------------------------------------------------------  60
19   ------------------------------------------------------------  60
20   ----------------------------F-------------------------------  60
21   ------------P-----------------------------------------------  60
22   ------------------------------------------------------------  60
23   ------------------------------------------------------------  60
24   ------------------------------------------------------------  60
25   ------------------------------------------------------------  60
26   -----------M------------------------------------------------  60
27   ------------------------------------------------------------  60

ATK  GPPGEKAPPRGETHRDRQRRAEEKRKRKKEREKEEEKQTAEYLKRKEEEKARRRRRAEKK  120
16   ------------------------------------------------------------  120
17   ------------------------------------------------------------  120
18   ----------------W---------Q---------------------------------  120
19   ---------------------------------------I--------------------  120
20   ----D-------------------------------------------------------  120
21   ---------C--------------------------------------------------  120
22   ------------------------------------------------------------  120
23   ------------------------------------------------------------  120
24   -------------------------------------------R----------------  120
25   ------------------------------------------------------------  120
26   -------------------Q----------------------------------------  120
27   ------------------------------------------------------------  120

ATK  AADVARRKQEEQERRERKWRQGAEKAKQHSARKEKMQELGIDGYTRQLEGEVESLEAERR  180
16   ---------------------------------------------------------D--  180
17   ------------------------------------------------------------  180
18   ------------------------------------------------------------  180
19   ------------------------------------------------------------  180
20   ------------------------------------------------------------  180
21   ------------------------------------------------------------  180
22   ------------------------------------------------------------  180
23   ------------------------------------------------------------  180
24   ------------------------------------------------------------  180
25   ------------------------------------------------------------  180
26   ------------------------------------------------------------  180
27   ------------------------------------------------------------  180

ATK  KLLQEKEDLMGEVNYWQGRLEAMWLQ  206
16   --------------------------  206
17   ----D---------------Q-----  206
18   --------------------------  206
19   --------------------------  206
20   ----D---------------Q-----  206
21   --------------------------  206
22   --------------------------  206
23   ----D---------------Q-----  206
24   --------------------------  206
25   --------------------------  206
26   --------------------------  206
27   --------------------------  206
```

FIG. 5. Amino acid sequences of HBZ protein in ATL cells with type 2 defective provirus. The predicted amino acid sequences (206 amino acids) of HBZ protein are shown. Case numbers are given on the left. The ATK-1 sequence is used as a control.

sequence is intact suggest that its expression is necessary for leukemogenesis. In particular, the finding that type 2 defective provirus lacking proviral polyadenylation signal sequences trapped a cellular polyadenylation signal suggests a critical role for *HBZ* in HTLV-1-induced oncogenesis. A recent study reported that HBZ protein is not necessary for in vitro immortalization of T lymphocytes but is critical for infectivity and persistence in vivo (4). Furthermore, this study indicates the significance of the *HBZ* gene in oncogenesis by HTLV-1, suggesting that persistent infection by HTLV-1 carrying the *HBZ* gene is essential for oncogenesis.

Retrovirus integrases generate short repeat sequences adjacent to an LTR during integration. It has been reported that human immunodeficiency virus with mutant integrase lacking activity still produces proviruses that are integrated into the host genome but exhibit no adjacent repeats (19), suggesting that cellular recombination and DNA repair machinery likely integrate the provirus. Therefore, the appearance of short repeats adjacent to LTRs indicates that viral integrase functions in integration. Since LTRs are conserved in integrated proviruses, they are thought to be essential for integration. Here we identified three kinds of type 2 defective provirus in ATL patients. In the first, the 5′ LTR was deleted and the internal proviral sequence was flanked by short repeat sequences at the

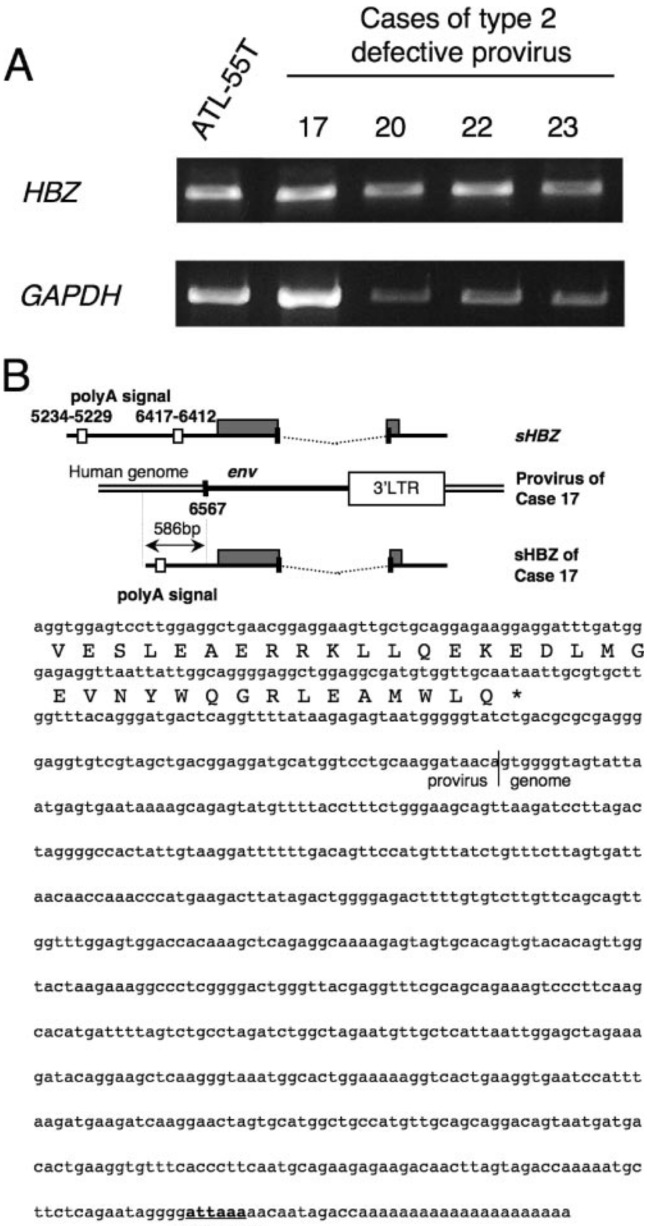

FIG. 6. *HBZ* transcription in ATL cells with type 2 defective provirus. (A) *HBZ* transcription in ATL cases with type 2 defective proviruses was analyzed by RT-PCR. *GAPDH* transcripts served as internal controls. RNA from ATL-55T cells served as a positive control for *HBZ* transcription. (B) 3′-RACE was undertaken to determine the polyadenylation signal in case 17. In that case, previously reported *HBZ* polyadenylation signals were deleted. 3′-RACE identified a polyadenylation signal (attaaa) (underlined) in the host genomic sequence. *sHBZ*, spliced form of the *HBZ* gene.

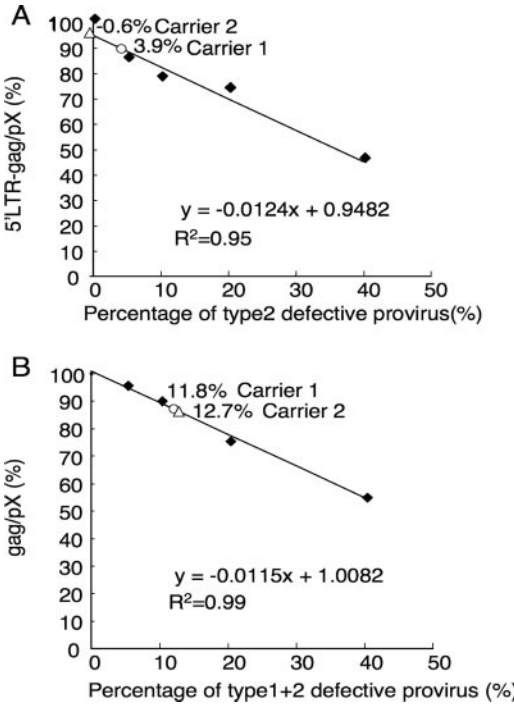

FIG. 7. Quantification of defective proviruses in carriers. To estimate the frequencies of type 1 and 2 defective proviruses in carriers, we quantified HTLV-1 provirus at three different provirus regions: 5′ LTR-*gag* (deleted in type 2 defective provirus), *gag* (deleted in both type 1 and 2 defective proviruses), and pX (conserved in all proviruses). Genomic DNA from an ATL patient with type 2 defective provirus was mixed with genomic DNA from an ATL cell line with a complete provirus (TL-Om1) in various proportions. These DNAs were used to quantify provirus, and regression lines were drawn using the least-squares method (solid diamond). The frequencies of type 2 and total defective proviruses in carriers were calculated from quantitative data at the 5′ LTR-*gag* and pX (A) or *gag* and pX (B) regions, respectively. The frequencies of defective proviruses in carrier 1 (open circle) and carrier 2 (open triangle) were analyzed. R^2, coefficient of determination.

5′ end of the provirus. In these cases, TG in *pol* or *env* sequences was ligated to short repeats, indicating that the viral integrase recognized TG dinucleotides in these internal sequences rather than TG at end of the LTR. In a second type, short 5′ regions of the 5′ LTR U3 (18 to 43 bp) were retained at the 5′ end of the provirus. In these cases, it is unclear whether deletion occurred before or after integration. In a third type, short repeat sequences were not seen at integration sites, and short host genomic sequences were also deleted. In these cases, CA dinucleotides were retained at the 3′ end of the LTR, indicating that the viral integrase recognized these sequences at the integration step and suggesting that deletion of the 5′ LTR occurred after integration. Such deletions may block Tax expression, enabling ATL cells to escape the host immune system.

Quantitative analyses of defective provirus indicate that the frequency of type 2 defective provirus (27.8%) in ATL is much higher than that seen in carriers (less than 3.9%), suggesting that HTLV-1-infected cells with type 2 defective provirus tend to become leukemic. The presence of type 2 defective provirus without short repeats supports the hypothesis that the provirus 5′ LTR is deleted during oncogenesis. Such cells would lose Tax expression and escape host immunosurveillance but still transcribe *HBZ*. Type 2 defective proviruses with short repeats are thought to be generated before integration. It is likely that such infected cells are selected during leukemogenesis. Thus, two mechanisms generating type 2 defective provirus increase its frequency in ATL cells.

Analyses of type 2 defective provirus can provide important information regarding the minimum components of the HTLV-1 viral sequence necessary for oncogenesis. We have reported that all ATL cases examined express *HBZ* and that *HBZ* RNA has growth-promoting activity, while *tax* expression was observed only in some (44). All type 2 defective proviruses analyzed in this study retained the *HBZ* coding sequence, and ATL cells with type 2 defective provirus expressed *HBZ* transcripts. Since viral integrase recognizes CA sequences inside the provirus as shown here, defective proviruses without a 3′ LTR could in theory be generated during integration. However, no such defective provirus was observed in ATL cells, indicating that only proviruses capable of *HBZ* expression are selected during oncogenesis. With regard to the *tax*, *p30*, and *rex* genes, seven cases analyzed here lacked the second exon (Fig. 3). At least two cases (cases 17 and 20) likely lost the second exon before integration. Without this exon, Tax cannot activate NF-κB and CREB (48), suggesting that these activities of Tax are not essential for oncogenesis. Type 2 defective provirus trapped the cellular promoters in 5 of 12 cases. In three of these cases, regulatory genes (*tax* and *rex*) and accessory genes could be transcribed, since they retained the second exon. In the remaining two cases, only *p12* and *p13* genes could be expressed. Taken together, regulatory genes and most of the accessory genes, which are transcribed from the 5′ LTR, were not transcribed in 9 of 12 cases with type 2 defective proviruses. However, all cases could transcribe the *HBZ* gene from the 3′ LTR.

Tax has been demonstrated to be oncogenic in transgenic animals (22, 23, 27). In these animals, tumor types depend on the *tax* promoter, indicating that Tax is oncogenic in different cell types. It has been reported that Tax can induce chromosomal instabilities (26, 30, 42), which along with its other functions, such as NF-κB activation and functional inactivation of p53, should play an important role in oncogenesis. Our previous (44) and present studies suggest that *HBZ* also functions in oncogenesis. In human papillomavirus-induced tumors, the E6 and E7 viral proteins cooperate in oncogenesis (34). It is likely that the *tax* and *HBZ* genes function cooperatively in HTLV-1-induced oncogenesis. Further studies are required to clarify the roles of the *tax* and *HBZ* genes in ATL.

Here we have demonstrated that a defective provirus lacking the 5′ LTR was generated before and after provirus integration. Detailed analyses of such defective proviruses also suggested that *HBZ* activity promotes oncogenesis by HTLV-1.

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